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Sequence-Based Analysis of Thermal Adaptation and Protein Energy Landscapes in an Invasive Blue Mussel (*Mytilus galloprovincialis*)

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Abstract

Adaptive responses to thermal stress in poikilotherms plays an important role in determining competitive ability and species distributions. Amino acid substitutions that affect protein stability and modify the thermal optima of orthologous proteins may be particularly important in this context. Here, we examine a set of 2,770 protein-coding genes to determine if proteins in a highly invasive heat tolerant blue mussel (*Mytilus galloprovincialis*) contain signals of adaptive increases in protein stability relative to orthologs in a more cold tolerant *M. trossulus*. Such thermal adaptations might help to explain, mechanistically, the success with which the invasive marine mussel *M. galloprovincialis* has displaced native species in contact zones in the eastern (California) and western (Japan) Pacific. We tested for stabilizing amino acid substitutions in warm tolerant *M. galloprovincialis* relative to cold tolerant *M. trossulus* with a generalized linear model that compares in silico estimates of recent changes in protein stability among closely related congeners. Fixed substitutions in *M. galloprovincialis* were 3,180.0 calories per mol per substitution more stabilizing at genes with both elevated d_N/d_S ratios and transcriptional responses to heat stress, and 705.8 calories per mol per substitution more stabilizing across all 2,770 loci investigated. Amino acid substitutions concentrated in a small number of genes were more stabilizing in *M. galloprovincialis* compared with cold tolerant *M. trossulus*. We also tested for, but did not find, enrichment of a priori GO terms in genes with elevated d_N/d_S ratios in *M. galloprovincialis*. This might indicate that selection for thermodynamic stability is generic across all lineages, and suggests that the high change in estimated protein stability that we observed in *M. galloprovincialis* is driven by selection for extra stabilizing substitutions, rather than by higher incidence of selection in a greater number of genes in this lineage. Nonetheless, our finding of more stabilizing amino acid changes in the warm adapted lineage is important because it suggests that adaption for thermal stability has contributed to *M. galloprovincialis*' superior tolerance to heat stress, and that pairing tests for positive selection and tests for transcriptional response to heat stress can identify candidates of protein stability adaptation.

Key words: allosteric function, Gibbs free energy, global warming, positive selection, protein stability, transcriptome.

Introduction

Temperature plays a central role in governing rates of physiological activities and determining the stabilities of

macromolecules such as proteins (Somero 1995; Hochachka and Somero 2002; Fields et al. 2015). Protein adaptation to temperature strongly influences an poikilothermic species'

competitive ability and plays an important role in determining where such species can survive and thrive (Sunday et al. 2011). There is growing concern that global climate change will have major effects on species' distributions (Occhipinti-Ambrogi 2007; Rahel and Olden 2008; Somero 2010). One manifestation of this risk is the spread of invasive species. The past few decades have seen an increase in invasive species establishment and persistence in northern latitudes (Ruiz et al. 2000; Stachowicz et al. 2002; Hellmann et al. 2008). Therefore, it is important to understand the physiological and molecular basis of species-specific differences in temperature tolerance, especially when studying differences between closely related native and invasive species (Watt and Dean 2000; Petit 2004; Gu and Hilser 2009; Somero 2010; Lockwood and Somero 2011a; Fields et al. 2015).

Blue mussels of the genus *Mytilus* are a promising study system for the investigation of differences in physiological tolerance among closely related native and invasive species at the molecular level because of their recent divergence and their similar effective population sizes, levels of polymorphism, and rates of molecular evolution (Ahmed and Sparks 1970; Seed 1992; Sarver and Foltz 1993; Comesana et al. 1999; Bierne et al. 2003; Cruz et al. 2005; Saarman and Pogson 2015). It is known that *Mytilus galloprovincialis* evolved in the Mediterranean and is unique among blue mussels in its ability to invade and outcompete native congeners in new localities. For example, *M. galloprovincialis* has successfully colonized California, British Columbia, Japan, Chile, New Zealand, the Auckland Islands, southern Australia and South Africa (Grant and Cherry 1985; Seed 1992; Heath et al. 1995; Geller 1999; Rawson et al. 1999; Robinson et al. 2007; Westfall and Gardner 2010; Borsa et al. 2012). Physiological differences between *M. galloprovincialis* and other blue mussels have been documented for cardiac performance, metabolic rates, enzymatic activity levels, and mortality rates, especially at temperatures between 26 °C and 32 °C (Buckley et al. 2001; Braby and Somero 2006; Tomanek and Zuzow 2010; Dowd and Somero 2013). Since high temperatures in this thermal range are common in intertidal habitats during low tides (Dowd and Somero 2013), these studies suggest that superior thermal resistance might contribute to *M. galloprovincialis*' capacity to outcompete the native *M. trossulus* in the southern extent of its distribution in Japan and California (Sarver and Foltz 1993; Gardner 1994; Suchanek et al. 1997; Rawson et al. 1999; Braby and Somero 2006; Somero 2010).

It is widely known that temperature has widespread effects on the functional rates and structural stabilities of proteins (Somero 1995; Zavodszky et al. 1998; Fields et al. 2015). Orthologous proteins of organisms adapted to different temperatures typically show differences in thermal responses in ligand-binding abilities, catalytic rates, and stabilities of higher order structures (Hochachka and Somero 2002). Recent studies of several proteins involved in cellular energy metabolism

have revealed that adaptive differences between orthologues of closely related species can result from small numbers of amino acid changes (Fields et al. 2006; Dong and Somero 2009; Lockwood and Somero 2012). These substitutions often modify the numbers and strengths of weak-bond interactions in the native folded protein, thereby modifying the protein's net Gibbs free energy of stabilization (ΔG_{stab}). However, the proteomic distribution of temperature-related adaptive substitutions such as those documented in metabolic proteins remains unknown (see Lockwood and Somero 2012). In the genus *Mytilus*, a study by Lockwood et al. (2010) used transcriptional response to investigate physiological traits involved in surviving acute heat stress, and found that 1,531 genes had responses to experimental change in temperature from 13 °C to 32 °C. However, only 96 of these had a response specific to heat tolerant *M. galloprovincialis*, suggesting that only a subset of genes involved in heat stress response are subject to species-specific thermal selection.

Here, we test predictions established from previous studies that compared 1) genome-wide patterns in thermophiles relative to mesophiles, 2) a small number of glycolytic enzymes in vitro in species inhabiting narrow ranges of temperature, and 3) transcriptional responses in warm and cold tolerant *Mytilus* congeners. Comparisons among species with stark differences in thermal range have indicated there is a genome-wide influence of temperature on both protein stability and amino acid content (Fields and Somero 1998; Zeldovich et al. 2007; Cherry 2010), but that stabilizing amino acid changes in thermophiles are concentrated in proteins involved in metabolism (Gu and Hilser 2009). On the other hand, studies comparing glycolytic enzymes in vitro have demonstrated that enzyme kinetic parameters (K_m and/or K_{cat}) can adapt to subtle changes in temperature (e.g., Graves and Somero 1982; Lockwood and Somero 2012; reviewed in Fields et al. 2015). Studies of transcriptional responses to heat stress indicate that only a small portion of the genome might be subject to temperature-related selection unique to the warm tolerant lineage (Lockwood et al. 2010). These previous studies lead to the prediction that different thermal environments can cause adaptive divergence in protein structure and function among closely related species, but that these differences might be limited to a small subset of the genome.

In this study, we investigate the importance of temperature-related selection on protein stability in contributing to the evolution of the invasive *M. galloprovincialis*' distinctive heat tolerance. We use in silico estimates of changes in Gibbs free energy of amino acid substitutions to test for more stabilizing changes in heat tolerant *M. galloprovincialis* relative to cold tolerant *M. trossulus*. We apply a generalized linear model to test this prediction, thereby accounting for phylogenetic history, other possible conflicting selective forces, and transcriptional response. We also test for enrichment in heat tolerant *M. galloprovincialis* of a priori thermally

sensitive GO terms identified in a comparative study of protein stability between thermophiles and mesophiles by Gu and Hilser (2009). Our findings contribute to understanding of the mechanisms of thermal adaptation, and have implications for the potential of species to track the narrow shifts in temperature predicted with global warming.

Materials and Methods

RNA Sequencing in *Mytilus*

For each species, we collected one young adult individual (7–10 mm shell length) from various worldwide localities for RNA extraction (table 1). *Trichomya hirsuta* was used as the out-group in all phylogenetic analysis. All mussels from abroad were dissected on the day of collection, preserved in RNAlater RNA Stabilization Solution (Life Technologies, Gaithersburg, MD), shipped on ice and stored in a -80°C freezer for a maximum of 3 months before RNA extraction.

Total RNA was extracted using the Agencourt RNAdvance Tissue Kit (Beckman Coulter, Brea, CA) following the manufacturer's instructions. We removed any gonads present in larger individuals to improve overlap in transcript capture, minced all remaining tissues using a razor blade on a glass slide, and used 15 μg for the RNA extraction. We measured RNA concentration on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Between 2,180 and 5,500 ng (table 1) were submitted for Illumina sequencing to Hudson Alpha Institute for Biotechnology (HAIB) in Huntsville, Alabama. HAIB performed mature mRNA isolation, complementary DNA library preparation, cluster generation, and Illumina sequencing. Each of six samples was barcoded with a multiplex identifier adaptor and sequenced under the Illumina HiSeq platform to yield ~ 20 million 50-mer paired-end reads per individual.

Reference *M. californianus* EST Database Open-Reading-Frame Prediction and Annotation

We used a Sanger sequenced expressed sequence tag (EST) library from *M. californianus* as a reference for transcript alignments, prediction of protein-coding regions, and annotations (Gracey et al. 2008) [accession numbers: ES7325872–ES738966; ES408175–ES387463]. Dr Brent Lockwood and colleagues kindly provided the *M. californianus* 13,082 gene EST library in a refined format that included only unique single-copy protein-coding genes, as determined by the PartiGene bioinformatics pipeline (Parkinson et al. 2004; Lockwood et al. 2010). We predicted the single most likely protein-coding open reading frame (ORF) per transcript and performed annotation of each transcript with TRANSDECODER, a Trinity plugin (Grabherr et al. 2011). The output from TRANSDECODER was parsed with a custom python script, which selected the strand, sense or antisense, that we used for each transcript based on maximum length.

This method produced 9,219 unique ORFs for the study, which we refer to here as “genes.” ORFs that were unlikely to be protein-coding were distinguished by two criteria: 1) elevation of d_N/d_S ratios >2 with the CODEML program of PAML v.4.5 (Yang 2007), and 2) no significant BLASTX, BLASTP, or protein domain search hit. These proteins were excluded from all further analyses.

Alignments to the Reference *M. californianus* EST Library

We trimmed raw reads and discarded Casava “filtered” flagged reads using the fastq_illumina_filter v1.0.1 using default parameters (http://cancan.cshl.edu/labmembers/gordon/fastq_illumina_filter/). We then applied a quality filter (Phred score >20 , length >40) with EU_UTIL's FastqMcf toolkit (Aronesty 2011) and visualized these results with FastQC (Andrews 2010). Between 11,086,567 and 32,328,073 (table 1) high-quality (mean quality Phred score of 39) short-paired Illumina reads (mean 40 bp in length) from *M. edulis*, *M. trossulus*, *M. galloprovincialis*, and *T. hirsuta* were aligned to the *M. californianus* EST library using Sequence Search and Alignment by Hashing Algorithm (SSAHA2) (Ning et al. 2001). SSAHA2 is an appropriate tool for species with high polymorphisms such as *Mytilus* species because of its flexibility in the degree of mismatches allowed (Li and Durbin 2010). The reference assembly hash table was generated using a K-mer length of 13 and a skip step of six. Paired-end reads were aligned to this using a skip step of six and “solexa” defaults.

A consensus alignment for each species was generated for each gene following the methods described in Kober and Pogson (2017). Unless specified, we performed data processing using BioPerl (Stajich et al. 2002), BioPython (Cock et al. 2009), the Newick Utilities (Junier and Zdobnov 2010), and the R statistical package (Ihaka and Gentleman 1996), with the Open Grid Scheduler (available at <http://gridscheduler.sourceforge.net/>) for job scheduling. Read mapping qualities <30 were discarded and only the top highest MAPQ scored reads in the case of duplicates were retained. Pileups were created with the PySAM (available at <http://code.google.com/p/pysam/>) interface to SAMTOOLS (Li et al. 2009) and sites with base quality scores <25 were discarded. Valid alleles were represented on >8 reads and a frequency >0.126 . Insertion and deletions were ignored. Polymorphic sites were retained in the alignments with IUPAC ambiguity codes. Between 9,957,638 and 27,983,710 high-quality (mean Phred score 39) short paired-end Illumina reads (mean 40 bp in length) from *M. edulis*, *M. trossulus*, *M. galloprovincialis*, *M. galloprovincialis*, and *T. hirsuta* mapped successfully to the *M. californianus* EST reference sequences (table 1). Overall coverage and percentage of sites covered averaged $73.6\times$ and 97.7%, respectively. We used a final alignment of 2,770 protein-coding genes >60 codons in length with ambiguous sites removed for subsequent phylogenetic and protein stability analyses.

Table 1

Summary of Transcriptomic RNA Sequence Data Collected

Species	Date	Locality	Coordinates	Total Reads ^a	Average Coverage	% Missing
<i>Mytilus galloprovincialis</i>	Feb, 2011	Oakland, CA	37.805°, −122.257°	17,845,567	68.9	1.67%
<i>Mytilus edulis</i>	Mar, 2012	Darling Marine Center, ME	43.935°, −69.581°	9,957,638	50.3	4.19%
<i>Mytilus trossulus</i>	Dec, 2010	Newport Harbor, OR	44.631°, −124.049°	17,285,811	70.1	1.51%
<i>Mytilus californianus</i>	N/A	CA	36.955°, −122.025°	N/A	N/A	N/A (0%)
<i>Trichomya hirsuta</i>	Oct, 2012	N Stradbroke Is, Australia	−27.503°, 153.401°	27,983,710	73.6	5.39%

^aTotal number of reads that mapped to *Mytilus californianus* reference expressed sequence tags.

Phylogenetic Analysis and Tests for Positive Selection

We applied a phylogenetic approach to overcome the challenge of phylogenetic nonindependence (Felsenstein 1985), an issue well recognized in the context of protein evolution (Graves and Somero 1982). PhyML v3.0 (Guindon et al. 2003) was used to create gene trees, with a GTR model of nucleotide evolution, “free rates” for each class from the data, optimized tree topologies, branch length, and rate parameters, and the best tree topology of nearest neighbor interchange and subtree pruning and regrafting search operations. The topology of the most commonly observed ML tree (fig. 1) was in concordance with the previously published phylogeny of this group (Vermeij 1991; Seed 1992; Samadi et al. 2007), and we use the best ML tree for each locus in subsequent analyses.

We tested for significantly elevated d_N/d_S ratios among orthologs against the null expectation of 1 using the PAML v.4.5 package (Yang 1997, 2007) prior to testing for the stabilizing or destabilizing effects of amino acid substitutions on protein stability. There were three reasons for adopting this approach. First, genes with elevated d_N/d_S ratios are candidates for positive selection and thus may identify genes experiencing thermal adaptation (Dasmeh et al. 2013). Second, protein coding genes experience a variety of potentially conflicting selective forces, with the strongest forces being driven by antagonistic coevolution (Hughes and Nei 1988; Murphy 1993; Civetta and Singh 1995; Swanson and Vacquier 2002; Civetta 2003; Schlenke and Begun 2003; Clark et al. 2006; *Drosophila* 12 Genomes Consortium 2007; Haerty et al. 2007; Kober and Pogson 2013). Consequently, these loci might experience elevated rates of stabilizing or destabilizing substitutions irrespective of adaptation to temperature. Finally, there is evidence that clients of heat-shock proteins have elevated d_N/d_S ratios (Lachowiec et al. 2013, 2015) making it important to account for interactive effects between estimates of protein stability and d_N/d_S ratios. Maximum likelihood tests for elevated d_N/d_S ratios were performed using the CODEML program in PAML v.4.5 (Yang 1997, 2007). For each locus, ancestral states were inferred with model 0, and then used to test for elevated d_N/d_S ratios above the null expectation of 1 with the improved log-likelihood ratio test, “test 2,” of Zhang et al. (2005). In this test, the null hypothesis is the branch-site model A where d_N/d_S ratio varies among branches and codons, with a total

of four site classes in the sequence: there are two site classes along the background lineages with ratios ω_0 or ω_1 fixed to 0 and 1, respectively, but along the lineage of interest (the foreground lineage), the null model has an d_N/d_S ratio ω_2 fixed to 1, and the alternative model has a constrained d_N/d_S ratio at $\omega_2 \geq 1$ (Zhang et al. 2005). Tests were performed for all four terminal branches leading to the extant *Mytilus* species. We corrected for multiple tests using a Bonferroni correction of the family-wise error rate for each branch tested for each protein (Lu and Guindon 2014).

Transcriptional Response to Heat Stress

Transcriptional response to heat stress was included in our analyses because genes with significant changes in expression level under experimental heat stress are candidates of physiological traits involved in thermal tolerance (Lockwood et al. 2010). Additionally, transcription patterns are correlated with both rates of molecular evolution and protein stability (Drummond et al. 2005; Drummond and Wilke 2008; Serohijos et al. 2012), which makes it important to account for these associations, irrespective of the direction of the association. For example, heat-shock proteins are predicted to have high transcriptional response to heat stress (Lockwood et al. 2010) but not elevated d_N/d_S ratios because of their high level of selective constraint. Clients of heat-shock proteins may be expected to have high d_N/d_S ratios (Lachowiec et al. 2013, 2015) but not a transcriptional response to heat stress. Proteins with thermally adaptive substitutions might be expected to have both a transcriptional response and elevated d_N/d_S ratios. We parsed transcriptional response data reported by (Lockwood et al. 2010) into two broad categories using the clustering algorithm of the “heatmap.2” function in the R package “ggplot2” (Wickham 2009). We scored all loci with significant differences in transcription level when held at 13 °C versus 32 °C as a response to heat stress, and any loci with no significant difference in transcription as showing no response to heat stress.

eEscape Site-Specific Estimates of Protein Stability in *Mytilus* Species

We estimated changes in Gibbs free energy of stabilization ($\Delta\Delta G_{stab}$) of each amino acid substitution along each terminal

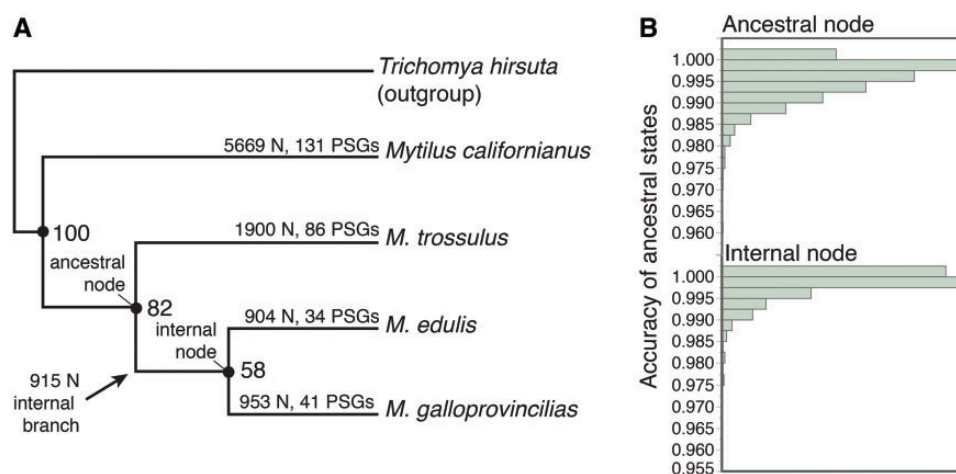


Fig. 1.—Phylogenetic results and ancestral reconstructions, showing (A) the cladogram of the most frequent tree obtained from the maximum likelihood analysis in PhyML v3.0 (Guindon et al. 2003), and (B) the distribution of the accuracy of all ancestral states predicted in the CODEML program of the PAML v.4.5 package (Yang 2007). Node labels represent the percent gene trees that support the presented topology, branch labels represent number of amino acid changes detected (N), and the number of positively selected genes detected (PSGs) from the positive selection analysis in the CODEML program of the PAML v.4.5 package (Yang 2007).

branch of the 2,770 gene trees (fig. 1A) using eEscape v2.1 (Gu and Hilser 2008). Adaptive divergence is predicted to increase protein stability at upper thermal extremes and increase protein flexibility (i.e., decrease stability) at lower thermal extremes. However, accurate description of protein thermodynamics is difficult because proteins are only marginally stable and exist as transient ensembles of conformational microstates (Wrabl et al. 2011). Thus, accounting for multiple subensembles is necessary but is computational difficult. eEscape v2.1 (Gu and Hilser 2008) overcomes this difficulty in silico by using amino acid sequence data to model the contribution of each residue to the stability constant, a metric representing the equilibrium of the natively folded and the multiple unfolded states of a protein (D'Aquino et al. 1996). Estimates of the contribution of each residue to the stability constant (ΔG_{stab}) are completed in a sliding window along the length of an amino acid sequence alignment. This sliding window approach allows for local estimates of ΔG_{stab} across stretches <20 residues in length, and makes eEscape an excellent tool for detecting subtle changes in protein thermodynamics such as those important in adaptive stabilizing substitutions (Fields et al. 2015). We estimated ΔG_{stab} of each residue of the 2,770 gene alignments, and calculated $\Delta\Delta G_{\text{stab}}$ as the difference between the ΔG_{stab} of each amino substitution (fig. 1A) and the ΔG_{stab} of the ancestral state inferred for the branch-sites tests from CODEML, which were all of high accuracy (fig. 1B): The overall mean posterior probability was 0.996 (± 0.004 SD; fig. 1B).

Generalized Linear Model to Detect Differences in $\Delta\Delta G_{\text{stab}}$ among Lineages

We tested the prediction of more stabilizing $\Delta\Delta G_{\text{stab}}$ mutations in heat tolerant *M. galloprovincialis* relative to cold

tolerant *M. trossulus* using a generalized linear likelihood model in JMP v11.0.0 (SAS Institute Inc 1989–2013) using $\Delta\Delta G_{\text{stab}}$ as the dependent variable and a full factorial set of independent variables, including the lineage of the amino acid substitution, results from the tests for elevated d_N/d_S ratios, and the transcriptional responses described in Lockwood et al.'s (2010) study. We investigated genes of interest with searches using the Protein Homology/analogY Recognition Engine v2.0 (Kelley and Sternberg 2009; Kelley et al., 2015), and mapped amino acid substitutions and estimates of ΔG_{stab} of *M. galloprovincialis*, *M. trossulus*, and the internal node onto the predicted protein structure.

Enrichment Analysis for A Priori GO Terms

We also tested for enrichment of GO terms at genes with elevated d_N/d_S ratios that showed shifts in protein stability in thermophiles relative to mesophiles in Gu and Hilser's (2009) study. Gene ontology (GO) terms (Gene Ontology Consortium 2004) were identified with the "trinotate" pipeline (available at <http://trinotate.sourceforge.net/>) based on a homology searches with NCBI-BLASTx and NCBI-BLASTp against the SWISSPROT database (version "Trinotate.Oct2013") with an e-value cutoff of 10e-5. We used a weighted algorithm that took the hierarchical structure of GO terms into account in the Bioconductor package topGO v2.14 (Gentleman et al. 2004; Alexa et al. 2006) and looked for overlap of these terms with a priori GO terms. The a priori GO terms included seven molecular function terms (enzyme regulator, translation regulator activity, protein transporter activity, ion transporter activity, helicase activity, catalytic activity, and structural molecule activity), a single biological process term (secretion), and two cellular component terms (nucleus and chromosome).

Table 2

Results of Branch-Site Tests for Positive Selection in PAML v.4.5 (Yang 2007)

Lineage	Total Sites Called ^a	Number of Genes Tested	Mean Length of Genes	Mean ω ^b	Number of PSGs ^c	Mean LRT ^d of PSGs
<i>Mytilus galloprovincialis</i>	1,639,598	1996	420.89	0.11912	40	7.2386
<i>M. edulis</i>	1,467,662	2046	435.88	0.1039	34	8.2249
<i>M. trossulus</i>	1,635,612	2117	442.50	0.1251	87	6.5140
<i>M. californianus</i>	1,897,333	2819	440.48	0.1277	130	6.6383
Overall	1,470,508	2770	434.94	0.1190	282	7.1539

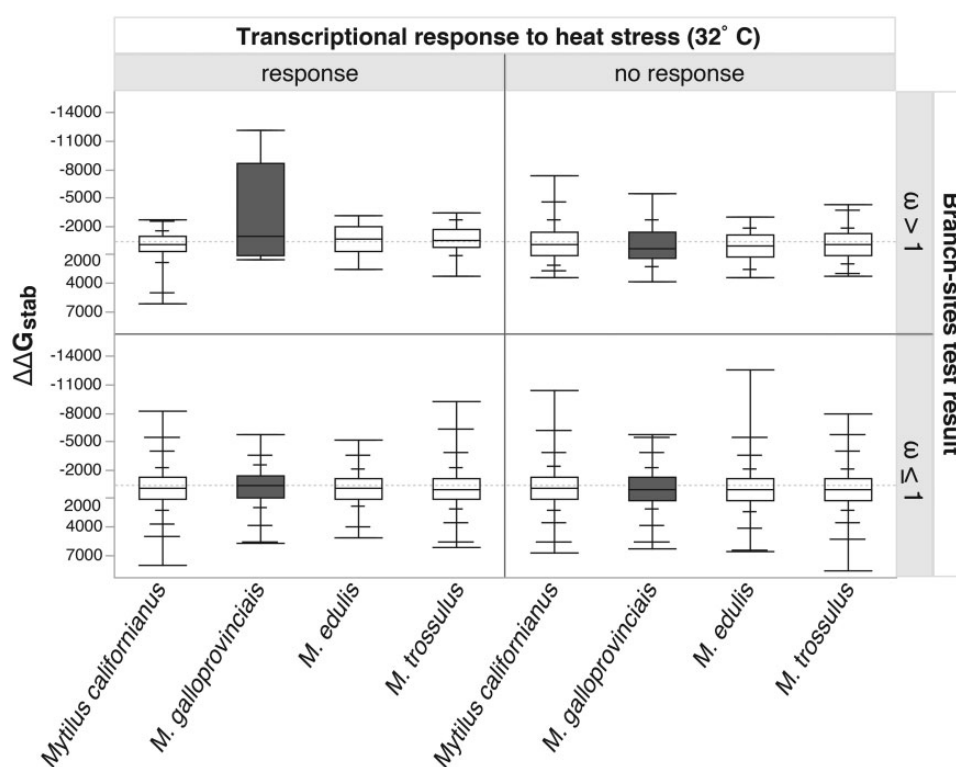
^aIn total, 631,194 of these sites that were called for all lineages were used in tests of positive selection.^bMean dN/dS ratio per gene for the lineage.^cPSGs, positively selected genes.^dLikelihood-ratio test score.

FIG. 2.—Quantile box-plots of mean eScale v2.1 estimates of change in Gibbs free energy of stability ($\Delta\Delta G_{stab}$) in calories per mol for each amino acid substitution identified in the 2,770 orthologs analyzed, separated by branch-sites tests for elevated d_N/d_S ratios (ω) and transcriptional response to heat stress at 32°C (Lockwood et al. 2010). $\Delta\Delta G_{stab}$ was estimated as the mean difference between the derived ΔG_{stab} and the ancestral ΔG_{stab} for each lineage for each substitution identified in the CODEML program of the PAML v.4.5 package (Yang 2007). More negative $\Delta\Delta G_{stab}$ values signify more stabilizing amino acid substitutions.

Results

Sequence-based estimates of protein energy landscapes in *Mytilus* spp. identified more stabilizing amino acid changes in heat tolerant *M. galloprovincialis* relative to cold tolerant *M. trossulus*, especially in genes with elevated d_N/d_S ratios and those with transcriptional responses to heat stress. This pattern was driven by $\Delta\Delta G_{stab}$ outliers in *M. galloprovincialis*. We did not obtain evidence that selection for protein stability was strong enough to enrich a priori candidates of thermal

sensitively in genes with elevated d_N/d_S ratios in *M. galloprovincialis* relative to other congeners.

Differences in $\Delta\Delta G_{stab}$ among Lineages

We identified 40 genes with significantly elevated d_N/d_S ratios in *M. galloprovincialis*, 34 genes in *M. edulis*, 87 genes in *M. trossulus*, and 130 genes in *M. californianus* (table 2; supplemental materials 1 and 2, Supplementary Material online). The greater number genes with elevated d_N/d_S ratios

Table 3

Summary of Generalized Linear Model Effect Sizes of Change in Gibbs Free Energy Constant ($\Delta\Delta G_{\text{stab}}$) by Lineage

	β^a of $\Delta\Delta G_{\text{stab}}$	SE	LRCS	P Value
By lineage				
<i>Mytilus californianus</i>	26.14	198.23	0.02	0.895
Internal branch	693.65	608.12	1.30	0.254
<i>M. galloprovincialis</i>	−705.80	263.95	7.15	0.008
<i>M. edulis</i>	36.89	68.28	0.29	0.589
By lineage by positive selection (PS)				
<i>M. californianus</i> by PS	79.74	199.80	0.16	0.690
Internal branch by PS	606.43	603.13	1.01	0.315
<i>M. galloprovincialis</i> by PS	−616.26	266.83	5.33	0.021
<i>M. edulis</i> by PS	0.00	N/A	N/A	N/A
By lineage by heat stress induced transcriptional response (HSITR)				
<i>M. californianus</i> by HSITR	229.12	198.23	1.34	0.248
Internal branch by HSITR	555.74	608.12	0.84	0.361
<i>M. galloprovincialis</i> by HSITR	−707.32	263.95	7.18	0.007
<i>M. edulis</i> by HSITR	−58.21	68.28	0.73	0.394
By lineage by PS by HSITR				
<i>M. californianus</i> by PS by HSITR	187.39	199.80	0.88	0.348
Internal branch by PS by HSITR	438.10	603.13	0.53	0.468
<i>M. galloprovincialis</i> by PS by HSITR	−615.77	266.83	5.32	0.021
<i>M. edulis</i> by PS by HSITR	0.00	N/A	N/A	N/A

Note.—Significant coefficients (β) indicated in bold. SE, standard error of the coefficient (β); LRCS, likelihood ratio χ^2 of the coefficient (β).

^aCoefficient of predicted $\Delta\Delta G_{\text{stab}}$ against *Mytilus trossulus* (full model P value = 0.0009).

detected in *M. trossulus* and *M. galloprovincialis* is consistent with previous work showing positive selection is more likely detected in longer branches (Nielsen et al. 2005; Kosiol et al. 2008; Kober and Pogson 2017), and suggests limited power to detect positive selection in *M. galloprovincialis* and *M. edulis* (fig. 1) within this data. Our generalized linear model found that amino acid substitutions in *M. galloprovincialis* were 3,180.0 calories per mol more stabilizing in a subset of 65 loci with both elevated d_N/d_S ratios and transcriptional response to heat stress (fig. 2), and 705.8 calories per mol more

stabilizing across all 2,770 loci investigated (table 3). The full model was highly significant (P value = 0.0009), and many individual parameter estimates were significant, notably in the *M. galloprovincialis* lineage (table 3). Removal of 26 *M. galloprovincialis* outlier orthologs with $\Delta\Delta G_{\text{stab}}$ outside of the 95% quantiles reduced the model to nonsignificance (P value = 0.1464).

Energy Profiles of Orthologs with Elevated d_N/d_S Ratios and Transcriptional Response to Heat Stress

Genes with transcriptional response to heat stress and significantly elevated d_N/d_S ratios in *M. galloprovincialis* fell within five contigs; MYC06848, MYC02156, MYC03882,

MYC07203, and MYC11426 (fig. 3). The identity and patterns of $\Delta\Delta G_{\text{stab}}$ within these genes suggested that substitutions within coiled regions of down-regulated proteins had the most stabilizing effects (fig. 3 and supplemental material 1, Supplementary Material online).

Uniform Enrichment of A Priori GO Terms

All a priori GO terms were equally common in all four *Mytilus* species analyzed. At candidate positively selected genes, we found 20 GO terms enriched in *M. galloprovincialis*, 86 in *M. edulis*, 66 in *M. trossulus*, and 51 in *M. californianus* (supplemental material 3, Supplementary Material online). Nearly all of these GO terms were low in the GO hierarchy (child terms) and only a single candidate GO term, “structural molecule activity,” was enriched in *M. trossulus*. When we compared the parent terms of this list, we found a much higher degree of overlap with those associated with thermal sensitivity in Gu and Hilser’s (2009) study, but that they were uniformly distributed across all four *Mytilus* species (fig. 4). Enriched a priori GO terms included “structural molecular activity,” “catalytic activity,” and “secretion” (fig. 4).

Discussion

Obtaining evidence for genome-wide selection on protein stability is particularly challenging, but recent computational approaches (Vertrees et al. 2005; Tehei et al. 2006; Gu and Hilser 2008; Li et al. 2012; reviewed in Somero et al. 2016) are a promising avenue forward (Benedix et al. 2009; Wrabl et al. 2011). The present study represents one of the first attempts to identify amino acid substitutions that stabilize protein structure in a species group known to have one lineage (*M. galloprovincialis*) that has undergone adaptation to warmer temperatures (Braby and Somero 2006; Lockwood et al. 2010; Somero 2010; Tomanek and Zuzow 2010; Lockwood and Somero 2011a). As expected, in silico estimates of $\Delta\Delta G_{\text{stab}}$ suggested stronger genome-wide selection on proteins for thermodynamic stability in heat tolerant *M. galloprovincialis* than in cold tolerant *M. trossulus*. More stabilizing amino acid substitutions had fixed in *M. galloprovincialis* relative to *M. trossulus* (table 3), and genes with both elevated d_N/d_S ratios and transcriptional response to heat stress had more stabilizing changes in *M. galloprovincialis* by $\sim 3,180$ calories per mol per substitution than in *M. trossulus* (fig. 2 and table 3). This level of $\Delta\Delta G_{\text{stab}}$ per substitution is in the range of documented adaptive stabilizing mutations in *Mytilus* species and in other systems (D’Amico et al. 2001; Dasmeh et al. 2013; Fields et al. 2015) and is above the level expected by random mutation (Tokuriki et al. 2007). This suggests that amino acid substitutions that account for significantly higher $\Delta\Delta G_{\text{stab}}$ in *M. galloprovincialis* represent an adaptive response to heat stress during its evolutionary history in the warm Mediterranean Sea (Thunell 1979).

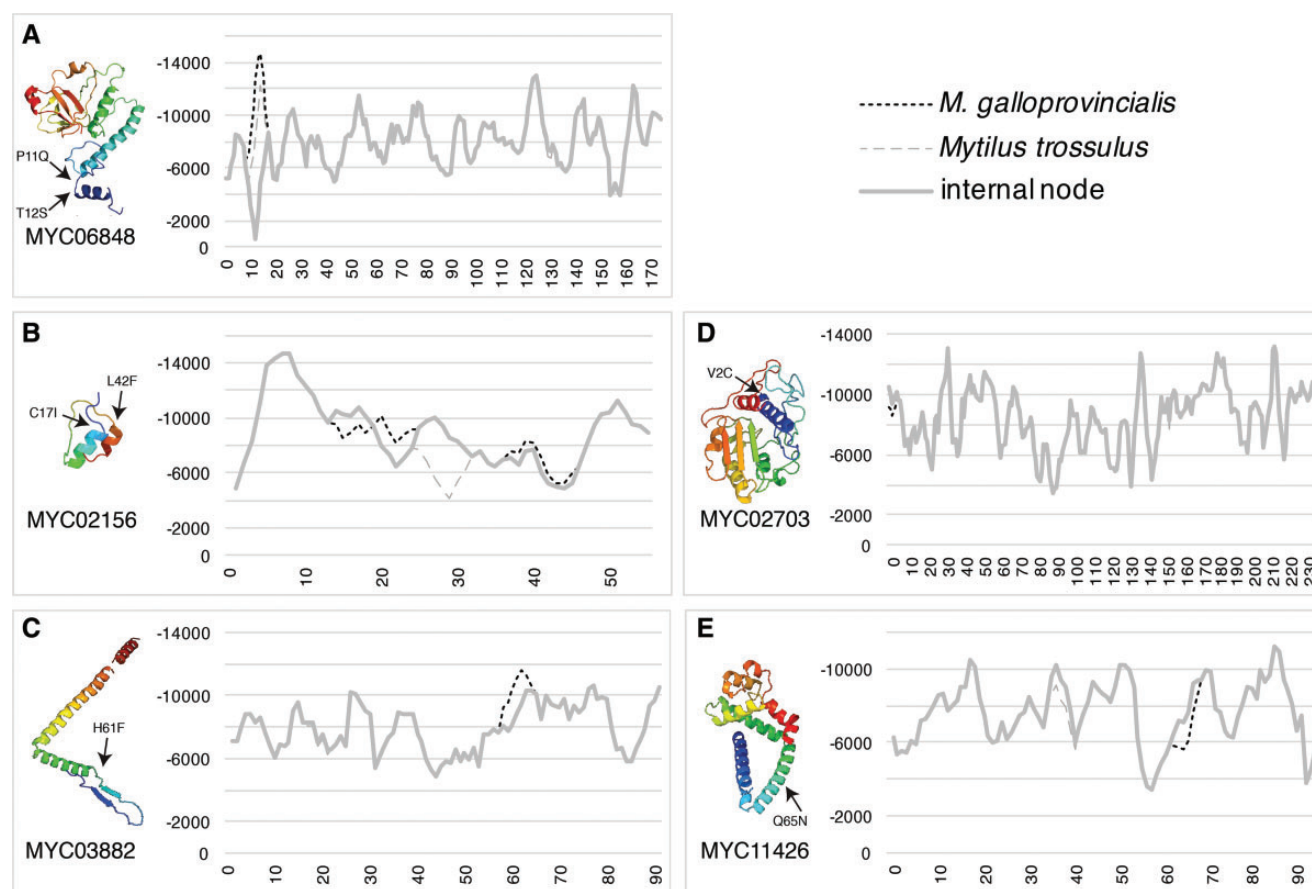


Fig. 3.—Protein structure predictions based on searches using the Protein Homology/analogY Recognition Engine v2.0 (Kelley and Sternberg 2009) and eScale v2.1 estimates of in Gibbs free energy of stability (ΔG_{stab}), in calories per mol, for loci with elevated d_N/d_S ratios (ω) and transcriptional response to heat stress in *Mytilus galloprovincialis*. Three genes exhibited clear evidence of stabilizing substitutions in *M. galloprovincialis*: (A) MYC06848, (B) MYC03882, and (C) MYC02156. Two genes showed minimal changes in energy profiles: (D) MYC02703, and (E) MYC11426. More negative ΔG_{stab} values signify higher protein stability. Protein structure image is colored by rainbow from red at the amine-terminus to blue at the carboxyl-terminus, with α -helices shown as helices, β -strands shown as arrows, and coils shown as narrow lines. See [supplemental material 1](#), Supplementary Material online, for details.

A priori tests for GO term enrichment revealed that selection for thermodynamic stability was not restricted to *M. galloprovincialis* but was present across all lineages. It is possible that difference in average $\Delta\Delta G_{stab}$ observed in *M. galloprovincialis* resulted from selection for stabilizing substitutions of greater effect, rather than selection acting on a greater number of genes. This pattern has been described previously for the evolution in myoglobin in cetaceans compared with terrestrial mammals (Dasmeh et al. 2013) and is supported by the importance of outliers in *M. galloprovincialis* in generating the signal of thermal adaptation. This hypothesis is also supported by the evolutionary and natural history of the group, which indicates significant overlap in thermal history (Seed 1992; Suchanek et al. 1997) and occasional exposure to thermal stress during tidal and weather extremes in all *Mytilus* species within their current distributions (Fields et al. 2006; Somero 2010; Dowd and Somero 2013).

The widespread signals of enrichment of a priori GO terms across all *Mytilus* species could, however, indicate

nonrepresentative a priori terms, insensitivity of the tests for positive selection, or lack of statistical power. The a priori GO terms used were based on thermophiles (Gu and Hilser 2009) and may not be appropriate for small shifts in thermal tolerance from 26 °C to 32 °C. For example, there are biases in amino-acid usage in thermophiles (Zeldovich et al. 2007; Cherry 2010) such that mesophiles may experience different patterns of constraint and positive selection. In addition, the a priori GO terms could represent vulnerability to any environmental stressor—such as oxidative, salinity, and radiation stress—rather than to thermal stress per se (Regoli and Principato 1995; Pampanin et al. 2005; Fasulo et al. 2008; Hamer et al. 2008; Lockwood and Somero 2011b; AlAmri et al. 2012; Trudeau et al. 2016) rendering them a priori more likely to produce significant branch-sites tests regardless of thermal differences (Oliver et al. 2010). Another possibility is that elevated d_N/d_S ratios may have been insensitive to the relevant substitutions, either because of general insensitivity of branch-sites tests to single amino acid changes

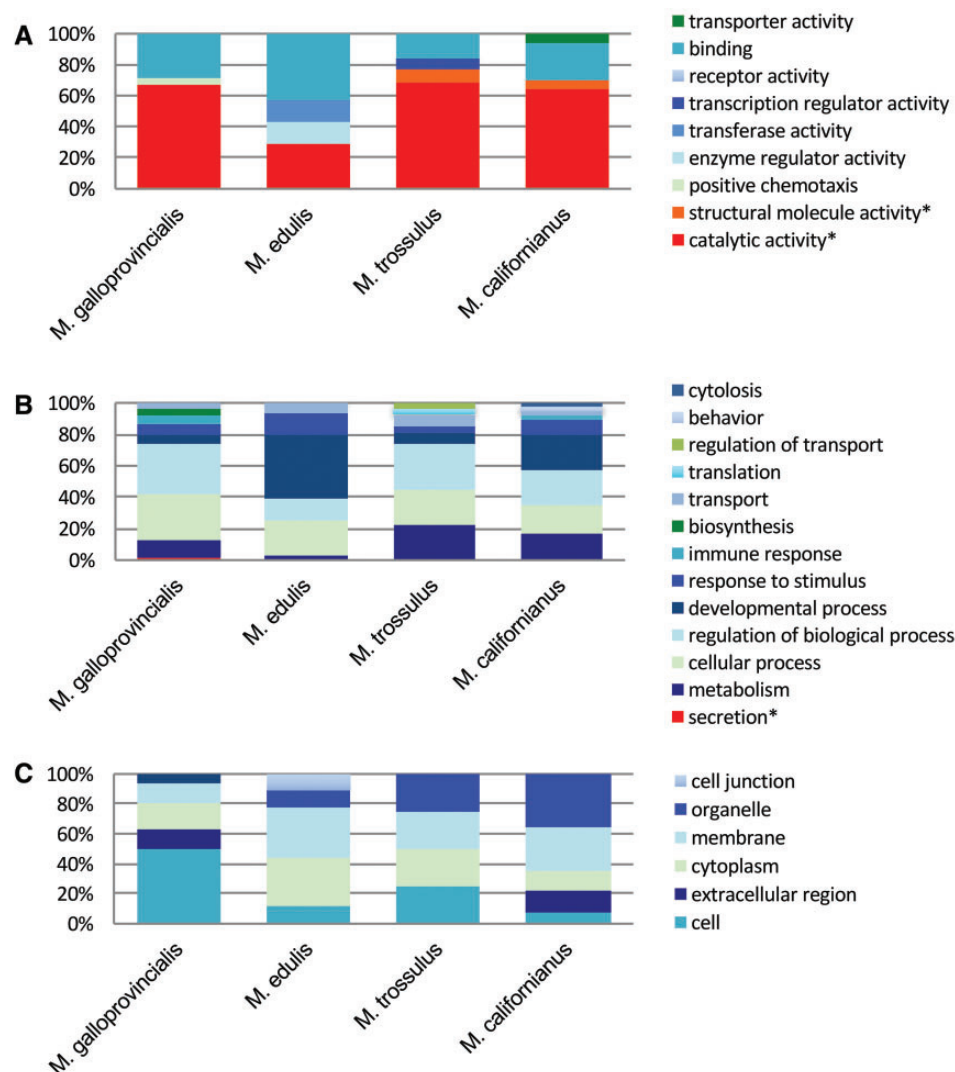


Fig. 4.—Frequency of top level GO terms in the list of enriched (A) molecular function, (B) biological process, and (C) cellular component GO terms based on topGO v2.14 (Gentleman et al. 2004; Alexa et al. 2006) analysis of lineage-specific positively selected genes in *Mytilus galloprovincialis*, *M. edulis*, *M. trossulus*, and *M. californianus*. Terms that overlap with those showing a strong thermal response in the Gu and Hilser (2009) study are marked with * and shown in warm colors.

(*Drosophila* 12 Genomes Consortium 2007; Ellegren 2008; Gharib and Robinson-Rechavi 2013; Lu and Guindon 2014) or because of the action of stronger selective agents such as antagonistic coevolution (Hughes and Nei 1988; Civetta and Singh 1995; Haerty et al. 2007). Indeed, many of the enriched GO terms are candidates for involvement in the innate immune response terms (e.g., immune response, response to stimulus, fig. 4) or sexual conflict (e.g., female germline ring canal formation, ovarian fusome organization, germ-line cyst formation, [supplemental material 3](#), Supplementary Material online). Finally, uniform enrichment could be due to lack of statistical power of our tests, which included only 34 to 130 genes with elevated d_N/d_S ratios, and only 2,770 genes total of the 13,759 ESTs available in the *M. californianus* EST library (Gracey et al. 2008). We suggest future research should aim

to increase the number of genes analyzed and, when available in the literature, attention should be directed at a priori genes and GO terms known to be involved in thermal adaptation in eukaryotic mesophiles.

The energy profiles of orthologs with both elevated d_N/d_S ratios and transcriptional response to heat stress in *M. galloprovincialis* (fig. 3) indicated that the largest changes in energy profile occurred in the expected direction (i.e., increasing stability) in warm tolerant *M. galloprovincialis* orthologs relative to cold tolerant *M. trossulus*. These changes tended to occur in coiled regions of local high stability, thus increasing the top peaks of stability of the whole protein (fig. 3A–C). Additionally, the largest stabilizing $\Delta\Delta G_{stab}$ estimates occurred in genes down-regulated when exposed to heat stress (fig. 3A and B). The changes in energy profiles of other

orthologs with both elevated d_N/d_S ratios and transcriptional response to heat stress were very minor, occurred within an α -helix or in up-regulated genes, and were unreliable (fig. 3D) or exhibited a mix of difference in ΔG_{stab} in the predicted and unpredicted directions (fig. 3E). These results suggest that adaptive stabilizing substitutions in *M. galloprovincialis* tended to occur within coiled regions of down-regulated genes with significantly elevated d_N/d_S ratios. However, a larger sample of genes with more comprehensive coverage of all open reading frames is needed to determine the generality of these patterns.

Conclusion

We observed significantly more stabilizing amino acid substitutions in *M. galloprovincialis* than in cold tolerant *M. trossulus* and suggest that these substitutions have contributed to its ability to tolerate heat stress several degrees warmer than its congeners. Our results provide confidence that it is possible to make in silico estimates of protein energy profiles using DNA sequence alone with the eScape method (Gu and Hilser 2008) in much smaller windows of thermal adaptation than originally tested (Gu and Hilser 2009). Adaptive shifts in protein energy landscapes appeared to be concentrated in a small subset of genes and tended to occur within coiled regions of heat stress induced down-regulated genes with significantly elevated d_N/d_S ratios.

Global warming has created concerns that poikilotherms will fail to evolve quickly enough to track rising temperatures. A key physiological response to a warming climate may be the fine-tuning of protein stability to maintain efficient allosteric function. Thermal adaptation in proteins is facilitated by the modulation of both local flexibility and protein-wide stability (Somero et al. 2016). However, the strength of the resulting selection for stability or flexibility is heretofore undocumented, and there are many other selection pressures important in sexually reproducing eukaryotes that may overpower any selection for protein stability. Our results suggest that adaptation of protein structure to the narrow shifts in temperature predicted with global warming may be an important component of species survival. However, our results also suggest that there may be some level of selection for thermodynamic stability across all *Mytilus* species, and that the higher level of stabilizing changes observed in *M. galloprovincialis* may have been caused by stabilizing substitutions of greater effect in the warm adapted lineage relative to the cold adapted lineage in a small subset of the genome. The remaining questions merit further study, and we suggest that future work targets a larger number of genes, especially those with both elevated d_N/d_S ratios and transcriptional response to heat stress, and should focus on experimental confirmation of the accuracy of in silico estimates of thermodynamic stability.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online at <https://doi.org/10.1093/gbe/evx190>.

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